REMARKS

Claims 146, 148-151, and 158-160 are pending. Claims 146, 148-151, and 158-160 are rejected under 35 U.S.C. § 112, first paragraph for new matter. Claims 146, 148-151, and 158-160 are rejected under 35 U.S.C. § 102(b) for anticipation by Keller et al. (U.S. Patent Publication No. 2002/0187548; hereinafter "Keller"). Claims 146, 148-151, and 158-160 are rejected under 35 U.S.C. § 102(a) for anticipation by Kodama et al. (Science 302:1223-1227, 2003) or Ferrando et al. (Blood 100:154A, 2002). By this reply, Applicant amends claims 146, 148, 150, and 151, cancels claim 158, adds new claims 161 and 162, and addresses each of the rejections.

Support for the Amendment

Support for the amendment to claim 146 is found in prior claim 158 and in pending claim 149. Support for the amendment to claims 148 and 150 is found in the specification at, e.g., ¶ [115].[1] Claim 159 is amended to correct claim dependency. Support for new claim 161 is found in Example 9 (¶¶ [0101]-[0106]). Support for new claim 162 if found in the Abstract and in, e.g., ¶¶ [0007] and [0010]. No new matter is added by the amendment.

Rejections under 35 U.S.C. § 112, first paragraph

The Office rejects claims 146, 148-151, and 158-160 under 35 U.S.C. § 112, first paragraph, for new matter, stating that the recitation of "isolated **adult** cells...in amended claim 146 represent(s) a departure from the specification and the claims as originally filed" (Office Action, p. 2; emphasis in original.) Applicant has amended independent claim 146 to remove the term "adult." The rejection of claims 146, 148-151, and 158-160 for new matter can now be withdrawn.

[1] The paragraph numbers refer to the patent application publication of the present application, U.S. Patent Publication No. 2007/0116688.

Rejections under 35 U.S.C. § 102(b)

Claims 146, 148-151, and 158-160 are rejected under 35 U.S.C. § 102(b) for anticipation by Keller. The Office states:

> Contrary to Applicant's assertion, it is noted that US Patent '548 does not limited [sic] Hox11(+) to only embryonic cells. US Patent '548 explicitly teaches that the present invention relates to novel population of precursor Hox11(+) cells that are capable of developing into different cell types (see paragraph 0011,0044 in particular).

(Office Action, p. 3).

To form the basis of a proper rejection under 35 U.S.C. § 102, a cited reference must disclose each and every element of the rejected claim. *See Lewmar Marine Inc. v. Barient Inc.*, 3 U.S.P.Q.2d 1766 (Fed. Cir. 1987) and Manual of Patent Examining Procedure (MPEP) § 2131.

Applicant has amended independent claim 146 to recite an isolated mammalian cell that endogenously expresses Hox11 and that lacks expression of CD45. Keller discloses only the transformation of embryonic cell populations with a Hox11 gene and fails to teach or enable the isolation of any cell that endogenously expresses Hox11. Thus, Keller fails to disclose each and every element of present claims 146, 148-151, and 159-162.

For example, at ¶¶ [0117] and [0118], Keller states that its embryonic cell population is immortalized by transformation with the Hox11 gene ("Another aspect of the present invention is a method to produce an immortalized precursor cell population by (a) transforming an embryonic stem cell population of the present invention with an immortalizing gene to create a transformed stem cell population...A preferred immortalizing gene of the present invention is a HOX11 gene."). Keller describes the transformation of its embryonic cell populations at Examples 13 and 14 (¶¶ [0212]-[0225]), and refers to five transformed cell populations prepared by these methods as EBHX-1, EBHX-4, EBHX-11, EBHX-14, and EBHX-15; these cells are characterized by their expression of cellular markers at, e.g., ¶¶ [0011] and [0134].

The Office refers to ¶ [0011] of Keller as evidence that Keller discloses the isolated cell of claim 146, and claims dependent therefrom. Yet it is clear that, when considered as a whole, Keller only discloses embryonic cell populations that are transformed with a Hox11 gene and fails to teach the isolation of a mammalian cell that endogenously expresses Hox11 and does not

express CD45, as is recited by present independent claim 146, and claims dependent therefrom (see M.P.E.P. § 2141.03 (VI) (emphasis in original); "A prior art reference must be considered in its entirety, i.e., as a whole, including portions that would lead away from the claimed invention.").

Finally, Keller fails to enable the isolation of a mammalian cell that endogenously expresses Hox11 and does not express CD45. In the field of molecular biology, courts have found that a specification may be deemed prima facie non-enabling for embodiments that are not specifically presented in the specification as working examples. *Ex parte Singh*, 17 U.S.P.Q.2d 1714, 1716 (Bd. Pat. App. & Int. 1990); *In re Marzocchi*, 439 F.2d 220, 223, 169 U.S.P.Q. 367, 169-170 (C.C.P.A. 1971). Here, Keller only describes transforming embryonic cell populations with a Hox11 gene. Nowhere does Keller teach the isolation of any cell that endogenously expresses Hox11 and does not express CD45.

For all the reasons discussed above, Keller fails to disclose each and every element of present claims 146, 148-151, and 159-162. This rejection should be withdrawn.

Rejections under 35 U.S.C. § 102(a)

Kodama

The Office also rejects claims 146, 148-151, and 158-160 under 35 U.S.C. § 102(a) for anticipation by Kodama. Kodama, which is the Applicant's own publication, was published on November 14, 2003, which is after the October 31, 2003, priority date of the present application.[2] Thus, Kodama is not prior art to the present application. This rejection should be withdrawn.

Ferrando

The Office also rejects claims 146, 148-151, and 158-160 under 35 U.S.C. § 102(a) for anticipation by Ferrando, stating that "Ferrando et al., teach an isolated adult cell expressing Hox11...It is noted that Ferrando et al., do not explicitly teach that said cells are CD45-[.] However, the reference and recited cells are the same as [sic] thus would inherently be CD45-" (Office Action, p. 4).

[2] Applicant notes that the priority data for this application, as shown on the Filing Receipt, is incorrect. Applicant concurrently submits a Petition to Correct Filing Receipt with this reply.

Anticipation requires that "each element of the claim at issue is found, either expressly described or under the principles of inherency, in a single prior art reference or that the claimed invention was previously known or embodied in a single prior art device or practice." *Kalman v. Kimberly-Clark Corp.*, 713 F.2d 760, 771 (Fed. Cir. 1983). Application of the doctrine of inherent anticipation requires that any information missing from the reference would nonetheless be known to be present in the subject matter of the reference when viewed by those of skill in the art. However, "anticipation by inherent disclosure is appropriate only when the reference discloses prior art that must *necessarily* include the unstated limitation, [otherwise the reference] cannot inherently anticipate the claims." *Transclean Corp. v. Bridgewood Servs., Inc.*, 290 F.3d 1364, 1373 (Fed. Cir. 2002) (emphasis in original). Thus, when a claim limitation is not explicitly set forth in a prior art publication, the extrinsic evidence put forth by the Office "must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill." *Continental Can Co. v. Monsanto Co.*, 948 F.2d 1264, 1268-1269 (Fed. Cir. 1991). Mere probabilities or possibilities that the missing material element or limitation is present in the prior art publication are insufficient for a finding of inherent anticipation. *Trintec Indus., Inc. v. Top-U.S.A. Corp.*, 295 F.3d 1292, 1295 (Fed. Cir. 2002).

One of skill in the art would not conclude that the cells of Ferrando are CD45-. Ferrando describes the expression of Hox11 in lymphoblasts obtained from patients having T-cell acute lymphoblastic leukemia (T-ALL) ("Expression levels of the oncogenic transcription factors *HOX11, HOX11L2, TAL1, BHLHB1* and *LYL1* were analyzed by quantitative real-time RT-PCR in leukemia samples from 24 and 30 adult T-ALL patients enrolled in ECOG and CALGB clinical trials, respectively."). Importantly, adult lymphoblasts are known to express CD45, and thus these cells are not CD45-, as suggested by the Office (see, e.g., Blank et al., J. Immunol. 166:6034-6040, 2001; Yaar et al., Am. J. Dermatopathol. 32:183-186, 2010; and Carulli et al., Leuk. Res. 32:263-267, 2007; copies of which are provided). Thus, Ferrando fails to teach, explicitly or inherently, a population of cells that are CD45(-), Hox11(+). Accordingly, Ferrando fails to teach each and every limitation of present claims 146, 148-151, and 158-160. This rejection should be withdrawn.

CONCLUSION

Applicant submits that present claims 146, 148-151, and 158-162 are in condition for allowance, and such action is respectfully requested.

Transmitted herewith is a petition to extend the period for replying for one month, to and including November 15, 2010, and authorization to deduct the fee required under 37 C.F.R. § 1.17(a) from Deposit Account No. 03-2095. If there are any other charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

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The Journal of Immunology

CD45 Tyrosine Phosphatase Controls Common {{gamma}}-Chain Cytokine-Mediated STAT and Extracellular Signal-Related Kinase Phosphorylation in Activated Human Lymphoblasts: Inhibition of Proliferation Without Induction of Apoptosis

Norbert Blank, Martin Kriegel, Thomas Hieronymus, Thomas Geiler, Silke Winkler, Joachim R. Kalden and Hanns-Martin Lorenz

J. Immunol. 2001;166;6034-6040
http://www.jimmunol.org/cgi/content/full/166/10/6034

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

CD45 Tyrosine Phosphatase Controls Common γ-Chain Cytokine-Mediated STAT and Extracellular Signal-Related Kinase Phosphorylation in Activated Human Lymphoblasts: Inhibition of Proliferation Without Induction of Apoptosis[1]

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The objective of this study was to test whether CD45 signals can influence signaling processes in activated human lymphoblasts. To this end, we generated lymphoblasts which proliferate in response to common γ-chain cytokines, but readily undergo apoptosis after cytokine withdrawal. In experiments with the CD45R0 mAb UCHL-1, but not control CD45 mAbs, we found significant inhibition of proliferation. Interestingly, the pan-CD45 mAb GAP8.3, which is most effective in inhibition of OKT-3-mediated proliferation in quiescent lymphocytes, was ineffective in lymphoblasts. Addition of CD3 mAb OKT-3 had no influence on IL-2-mediated proliferation (with or without UCHL-1). In contrast, after addition of OKT-3 to IL-4- and IL-7-stimulated proliferation assays, UCHL-1 signals could not significantly alter cellular proliferation. We did not find induction of apoptosis following CD45R0 signaling. In Western blots using mAbs detecting phosphorylated STAT-3, STAT-5, STAT-6, or extracellular signal-related kinase 1/2, we found that CD45R0 signaling could effectively diminish phosphorylation of these intracellular signaling components. Using RT-PCR, we found that CD45R0 signaling inhibited IL-2 mRNA production without major influence on IL-13, IL-5, or IFN-γ mRNA levels. Costimulation with OKT-3 and IL-2 optimally induced secretion of IFN-γ, TNF-α, and IL-5, which was not decreased by CD45 signals. In conclusion, we illustrate that CD45R0 signals control early cytokine receptor-associated signaling processes and mRNA and DNA synthesis in activated human lymphoblasts. Furthermore, we show the existence of CD45 epitopes (GAP8.3), which are active and critical for signaling in quiescent lymphocytes, but are nonfunctional in activated human lymphoblasts. *The Journal of Immunology,* **2001, 166: 6034–6040.**

The precise physiological significance of the most abundant surface protein on leukocytes, CD45 (leukocyte common Ag) still eludes definition. We and other investigators reported CD45 mAbs to be inhibitory to T and B lymphocyte proliferation (1–6) as well as basophil degranulation (7). On the other hand, CD45 expression also seems to be an absolute prerequisite for T and B lymphocyte activation (8–14). Perhaps the major contender for transduction of intracellular signals following CD45 cross-linking is the phosphotyrosine phosphatase enzyme activity located in the intracellular domain of CD45 (15–18). This 705 aa domain apparently causes constitutive dephosphorylation of its substrate $p56^{lck}$ at Tyr^{505}, leading to its subsequent activation (19, 20). Moreover, in CD45-negative cell lines decreased $p59^{fyn}$ activity was described (21). Retransfection of CD45 into these cells leads to $p56^{lck}$ activation. This was coupled to increased tyrosine phosphorylation and augmentation of phospholipase Cγl activity, resulting in Ca^{2+} flux (21–24) and the activation of protein kinase C (PKC).[4] Desai et al. and Majeti et al. (25, 26) have shown that dimerization of CD45 (after binding of epidermal growth factor in epidermal growth factor receptor-CD45 chimera (25, 26), binding of mAb) switches phosphatase activity off, leading to autophosphorylation of the negative regulatory Tyr^{505} and subsequent deactivation of $p56^{lck}$. However, this notion has recently been challenged by the finding that in thymocytes of CD45 knockout mice $p56^{lck}$ is hyperphosphorylated at its positive regulatory Tyr^{394}, with its activity being elevated 2- to 4-fold (27, 28). Some investigators have also reported costimulatory rather than inhibitory properties of different CD45 mAbs upon T cell (29, 30) and B cell activation (31), again highlighting epitope specificity of CD45 signaling processes.

CD45 seems to play an important role in coupling the TCR to the phosphatidyl inositol pathway (14). Cross-linking of CD45 with certain mAbs diminishes both inositol phosphate formation and Ca^{2+} flux upon stimulation with CD3/TCR or CD2 (8, 22, 23, 32–34), again suggesting a major role for CD45 in regulating early intracellular signaling processes in lymphocytes. Recently, an association of CD45 to lipid microdomains as major platforms regulating signal transduction has been discussed (35). In this model, CD45-stimulated cells release activated $p56^{lck}$ into the microdomain (dephosphorylated on Tyr^{505}), enabling signal transduction through the TCR-CD3-CD4 complex (35, 36). A prerequisite for this model is that CD45 is excluded from, but still is very close to,

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Received for publication February 22, 2000. Accepted for publication March 9, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the German Research Association (Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 263) and the Bundesministerium fur Bildung, Wissenschaft, Forschung und Technologie (to H.-M.L.).

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[4] Abbreviations used in this paper: PKC, protein kinase C; γ_c, common γ-chain; ERK, extracellular signal-related kinase; PI, propidium iodide; Jak, Janus kinase.

0022-1767/01/$02.00

the lipid microdomain. This represents a new mechanistic model which might help explaining the partially contradictory findings in CD45 signaling.

In contrast to results associating decreased PKC activity and T cell inactivation with CD45-transduced signaling, our previous findings suggest an additional role for CD45-triggered signaling, namely, cellular adhesion. We observed that triggering of PBMCs with certain mAbs to CD45, CD45RA, or CD45R0 leads to LFA-1/ICAM-1-dependent, heterotypic cellular aggregation (3). The CD45-associated phenomena occur independent of PKC activation and appear to be linked to subsequent activation of cAMP/cGMP-dependent kinases (3, 37). We could demonstrate (38) that triggering CD45 through certain epitopes induces intracellular cAMP accumulation and activation of protein kinase A in a dose- and time-dependent fashion. This cAMP synthesis did not occur in purified resting T cells and required presence of viable monocytes. Blockade of T cell-monocyte interactions with ICAM-1 mAb could prevent cAMP synthesis (38).

In this paper, we investigated the role of CD45 signaling in activated human lymphoblasts. We herein describe that signals through CD45R0 can inhibit proliferation and mRNA synthesis after stimulation with common γ-chain (γ_c) cytokines. Studying intracellular signaling events we found that cosignaling through CD45R0 specifically inhibits IL-2-triggered phosphorylation of STAT-3, STAT-5, and extracellular signal-related kinase (ERK) 1/2, and IL-4-stimulated phosphorylation of STAT-3 and STAT-6. These data associate γ_c cytokine receptor signaling events with CD45-regulated intracellular signaling processes in lymphoblasts and again highlight the dominant role for CD45 in influencing early cellular activation steps in human lymphocytes.

Materials and Methods

Cell preparation

PBMCs were isolated from heparinized peripheral blood by Ficoll-Hypaque (BAG, Lich, Germany) density gradient centrifugation, washed twice with PBS, and resuspended in RPMI 1640 (Life Technologies, Eggenstein, Germany) supplemented with 4 mM L-glutamine, 10 mM HEPES buffer, 100 U/ml penicillin, 0.1 mg/ml streptomycin (all BioWhittaker, Verviers, Belgium), and 10% (v/v) heat-inactivated FCS (Life Technologies). For generation of lymphoblasts, freshly isolated PBMCs were activated with 1 μg/ml PHA (Sigma, Deisenhofen, Germany) for 5 days with medium being replenished every 2–3 days. Thereafter, cells were expanded with 10 U/ml IL-2 (Boehringer Mannheim, Mannheim, Germany) for another 2–3 days. Resulting lymphoblasts were >95% $CD3^+$ (with 60–80% $CD4^+$ cells). Finally, lymphoblasts were extensively washed and cultured as indicated.

Abs and reagents (final concentration)

IL-2 (10 U/ml) was purchase from Boehringer Mannheim. IL-4, IL-7, and IL-15 (10 ng/ml) were purchased from PeproTech EC (London, U.K.). Hybridoma cells producing CD3-mAb OKT-3 (IgG2a, 1 μg/ml), pan-CD45 mAb NIH45-2 (IgG1, 10 μg/ml), or pan-CD45 mAb GAP8.3 (IgG2a, 10 μg/ml) were obtained from American Type Culture Collection (Manassas, VA). Hybridoma cells producing CD45R0 mAb UCHL-1 (IgG2a, 10 μg/ml) were obtained from Peter Beverly (University College, London, U.K.). mAbs were purified from hybridoma cell supernatant with the fast protein liquid chromatography unit LCC 500 plus (Pharmacia, Erlangen, Germany) using HiTrap protein A-Sepharose columns from Pharmacia. CD45RA mAb HB-11 (IgG1, 10 μg/ml) was a generous gift from J. Byrne (University of Alabama, Birmingham, AL). CD2 mAbs AICD2.M1 and M2 (1 μg/ml) were a generous gift B. Schraven and S. Meuer (University of Heidelberg, Heidelberg, Germany). Staphylococcal enterotoxin B (10 ng/ml) was purchased from Sigma. pERK-1/2 mAb (E-4) and ERK mAb (K-23) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). pSTAT-3, pSTAT-5, and STAT-5 mAbs were purchased from Upstate Biotechnology (Lake Placid, NY). pSTAT-6 was purchased from New England Biolabs (Beverly, MA). mAbs recognizing STAT-3 and STAT-6 were obtained from Transduction Laboratories (Lexington, KY).

Quantification of apoptosis

Microscopic examination of the cell cultures prone to undergo apoptosis revealed morphological changes like zeiosis. For quantification of apoptosis, DNA staining with propidium iodide (PI; Sigma) and flow cytometry analysis were performed as previously described (39). In brief, 4×10^5 cells were pelleted with 200 × *g* and gently resuspended in 150 μl of hypotonic fluorochrome solution of 50 μg/ml PI in 0.1% (w/v) sodium citrate plus 0.1% (v/v) Triton X-100 (Sigma). After a minimum period of 6 h in the dark at 4°C, samples were analyzed on a FACScan (Coulter, Hialeah, FL). The percentage of apoptotic cells was calculated as follows: percentage of cells with subdiploid DNA content × 100 divided by percentage of all cells positive for PI staining.

Cell proliferation assays

[^{3}H]Thymidine uptake and incorporation into genomic DNA was used to quantify cellular proliferation. PHA blasts (1×10^5) were incubated for 72 h in a total volume of 150 μl of medium in a 96-well round-bottom microtiter plate (Costar, Cambridge, MA) with the indicated reagents in triplicates at 37°C and 5% CO_2. Samples were pulsed for the last 6 h with 0.5 μCi/well [^{3}H]thymidine (2 Ci/mmol; Amersham, Braunschweig, Germany) and harvested onto glass fiber filters. Radioactivity was measured by liquid scintillation counting.

Western blot analysis

IL-2-expanded PHA blasts were extensively washed and aliquots of 1×10^6 cells were cultured for 8 h in medium. Thereafter, UCHL-1 mAb was added for 10 min before IL-2, IL-4, or OKT-3 were pipetted to culture medium as indicated. After another 10–20 min, cells were lysed in radioimmunoprecipitation assay lysis buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 5 mM EDTA, 0.5% (v/v) Nonidet P-40, 1% (v/v) Triton X-100, 1 mM sodium vanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 0.25% sodium deoxycholate at 4°C for 1 h (all reagents from Sigma) Supernatants were clarified by centrifugation for 10 min at 10,000 × *g* at 4°C. Protein concentration was determined using a Bradford method protein assay kit (Bio-Rad, Munich, Germany). Twenty micrograms of total protein lysates from each sample was diluted in an equal volume of 2× SDS sample buffer, boiled for 5 min, and resolved by SDS-PAGE. Immunoblots were performed by semidry transfer from the proteins onto nitrocellulose membranes. Unspecific binding sites were blocked in freshly prepared 5% nonfat dry milk in PBS/0.1% Tween 20 for at least 30 min at room temperature. A total of 0.1–1 μg/ml of the specific Ab as indicated was incubated in blocking buffer overnight at 4°C. Subsequently, 0.2–1 μg/ml HRP-conjugated goat anti-mouse (or anti-rabbit) IgG was added in blocking buffer for 1.5 h at room temperature. Detection was performed using ECL (Amersham). After detection of phosphorylated forms of proteins, Abs were washed off the membranes by incubation in 62.5 mM NaCl, 100 mM 2-ME, and 2% SDS for 30 min at 50–60°C. After washing in PBS/0.1% Tween 20, membranes were blocked as described above, and staining with Abs specific for all forms of signaling molecules was performed as described.

mRNA semiquantification by RT-PCR

To analyze the expression of apoptosis-related gene products, lymphoblasts were lysed 6–8 h (some variation in between the experiments) after stimulation with the reagents as indicated, and total RNA was isolated using a Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. mRNA purification included DNase digestion on the column following the manufacturer's recommendations. mRNA was reverse transcribed with oligo(dT) primers and amplified with gene-specific primers as described elsewhere (40). The gene-specific primers (all intron spanning) and PCR conditions were as follows:

GAPDH upstream, 5′-GCAGGGGGGAGCCAAAAGGG-3′; downstream, 5′-CCATGGACGGTCGGGGTCGCAGTTTC-3′ (60°C for 1 min, 72°C for 1 min); IL-2 upstream, 5′-ATGTACAGGATGCAACTCCTG TCTT-3′; downstream, 5′-GTTAGTGTTGAGATGATGCTTTGAC-3′ (60°C for 1 min, 72°C for 1 min); IL-4 upstream, 5′-ATGGGTCTCACC TCCCAACTGCT-3′; downstream, 5′-CGAACACTTTGAATATTTCTC TCTCAT-3′ (60°C for 3 min); IL-5 upstream, 5′-GCTTCTGCATTT GAGTTTGCTAGCT-3′; downstream, 5′-TGGCCGTCAATGTATTTCTT TATTAAG-3′ (60°C for 3 min); IL-12 (40-kDa) upstream, 5′-TAATCG TCCAAAAGTGTTA-3′; downstream, 5′-TTCTGTTCTGCTGTGTCT TG-3′ (60°C for 3 min); IL-13 upstream, 5′-ATCACCCAGAACCAGA AG-3′; downstream, 5′-GAACCGTCCCTCGCGAAAAAGT-3′ (60°C for 1min, 72°C for 1 min); TNF-α upstream, 5′-ATGAGCACTGAAAGCATGA TCCGG-3′; downstream, 5′-GCAATGATCCCAAAGTAGACCTGCCC-3′ (60°C for 1 min, 72°C for 1 min); IFN-γ upstream, 5′-ATGAAATATA

CAAGTTATATCTTGGCTTT-3′; downstream, 5′-GATGCTCTTCGAC CTCGAAACAGCAT-3′ (60°C for 3 min); TNFR p60 upstream, 5′- TGT GTCTCCTGTAGTAACTG-3′; downstream, 5′-ACGAATTCCTTCCAG CGCAA-3′ (60°C for 1min), 72°C for 1 min); TNFR p80 upstream, 5′-AAGTCCCTGACTCTGTGTGA-3′; and downstream, 5′-TCCTGAGTCAA CTTGAAGGA-3′ (60°C for 1 min, 72°C for 1 min).

cDNA was amplified for 28 and 35 cycles (21 and 28 cycles for GAPDH). After agarose gel electrophoresis, PCR products were visualized under UV light, and band intensities of the corresponding cycles were graded as described elsewhere (40).

Measurement of cytokines in culture supernatants

Cytokines in culture supernatants were measured using ELISA kits from R&D Systems (for IL-5, IFN-γ, and TNF-α; Wiesbaden, Germany). For measurement of IL-2, we used capture anti-IL-2 Ab MAB602 and biotin-linked detection Ab BAF202 (both from R&D Systems) along with streptavidin-peroxidase and peroxidase substrate (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's recommendations. Quantification of IL-4 was performed with capture anti-IL-4 Ab 8D4-8 and biotin-linked detection Ab MP4-25D2 (both from PharMingen, Hamburg, Germany) along with streptavidin-peroxidase and peroxidase substrate (Boehringer Mannheim) according to the manufacturer's recommendations.

Statistical analysis was performed using a paired or unpaired (where appropriate) nonparametric Wilcoxon test.

Results

In freshly isolated PBMCs, pan-CD45 mAbs NIH45-2 and GAP8.3 as well as CD45R0 mAb UCHL-1, but not CD45RA mAb HB11, strongly inhibited cellular proliferation in response to various stimuli like CD3 mAb OKT-3 (Fig. 1), superantigen staphylococcal enterotoxin B, or CD2 mAbs (data not shown). These data show that all of these mAbs with the exception of HB11 bind to CD45 epitopes with signaling capacities. To study the influence of CD45 signals on activated human lymphocytes, we generated lymphoblasts as described in *Materials and Methods*. These cells will readily proliferate specifically after activation with γ_c cytokines IL-2, IL-4, IL-7, or IL-15 (Fig. 2). In contrast, cytokine withdrawal induces programmed cell death (41). Incubation with pan-CD45

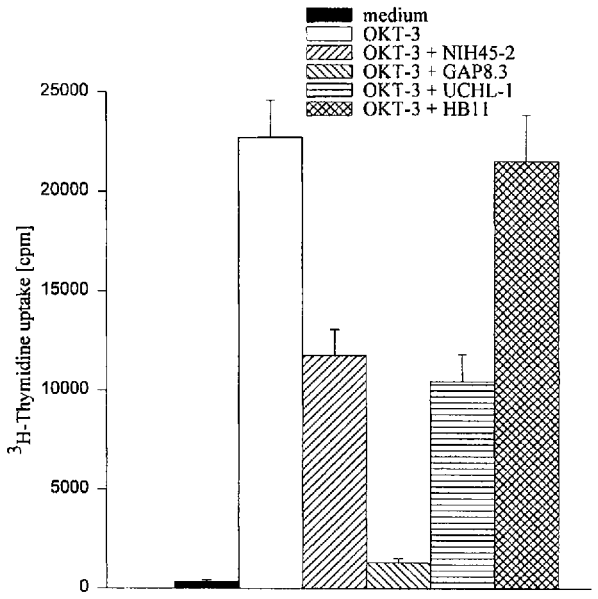

FIGURE 1. CD45 mAbs inhibit proliferation in quiescent PBMCs. PBMCs were isolated as described in *Materials and Methods*. One × 10^5 cells/well were stimulated with the reagents as indicated in triplicates. DNA synthesis was quantified as specified in *Materials and Methods*. Numbers are mean values ± SD of 10 different experiments. CD45 mAbs alone did not induce proliferation over background levels.

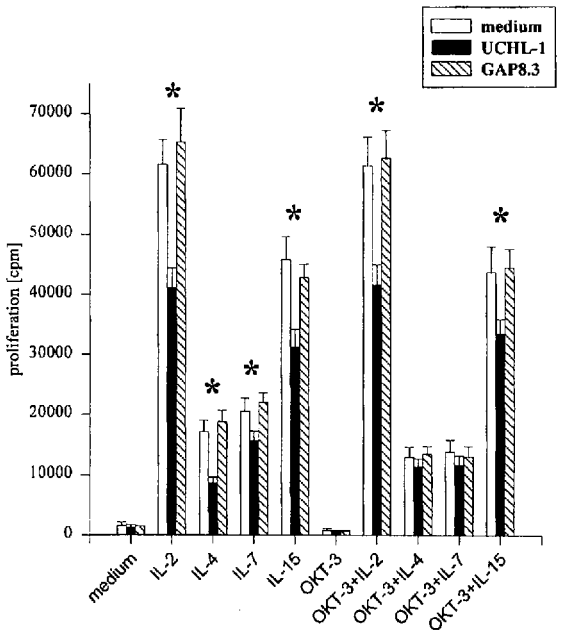

FIGURE 2. CD45R0 mAb UCHL-1 inhibits γ_c cytokine-driven proliferation of lymphoblasts. Lymphoblasts were generated as described. One × 10^5 cells/well were stimulated with the reagents as indicated in triplicates. DNA synthesis was quantified as specified in *Materials and Methods*. Numbers are mean values ± SD of 10 different experiments. *, $p < 0.05$, comparing [^{3}H]thymidine incorporation in medium vs UCHL-1 mAb-containing wells within each experimental setting.

mAbs NIH45-2, GAP8.3, or CD45R0 mAb UCHL-1 alone did not lead to proliferation. Importantly, however, UCHL-1, in contrast to GAP8.3 or NIH45-2 (data not shown), significantly inhibited proliferation triggered by IL-2, IL-4, IL-7, or IL-15 (Fig. 2). Furthermore, stimulation of lymphoblasts with CD3 mAb OKT-3 alone did not trigger proliferation (Fig. 2). Interestingly, however, cosignaling through CD3 and CD45R0 diminished UCHL-1-mediated inhibition of IL-4- or IL-7-triggered proliferation (Fig. 2), whereas IL-2- and IL-15-dependent DNA synthesis was practically unaffected by OKT-3 (Fig. 2). This pattern held through for all concentrations of cytokines tested (1–10 U/ml or ng/ml, respectively, data not shown).

Since lymphoblasts are prone to undergo apoptosis upon various stimuli, especially those limiting proliferation, we wondered whether this UCHL-1-mediated inhibition of proliferation consequently induced apoptosis, or vice versa was the effect of increased apoptosis as seen after cytokine withdrawal. To this end, we compared percentages of apoptosis under various concentrations of IL-2 or IL-4. Table I shows that suboptimal concentrations of IL-2 not only lead to decreased DNA synthesis (Fig. 2), but subsequently also to increased rates of apoptosis. Importantly, however, addition of UCHL-1 or GAP8.3 did not induce apoptosis over the quantities seen in cytokine-stimulated lymphoblasts in the absence of Abs (Table I). Similar results were seen when we tested various concentrations of IL-4 in the identical experimental setting (data not shown).

For optimal signal transduction after γ_c cytokine stimulation, tyrosine phosphorylation and subsequent activation of several proteins like STAT-3, STAT-5, ERK1/2, or STAT-6 (IL-4 STAT) is required (reviewed in Refs. 42 and 43). These early signaling proteins are regulated in their activity via tyrosine phosphorylation

Table I. *Percentage of apoptotic lymphoblasts after CD45 signaling*[a]

		IL-2			
	Medium	1 U/ml	2 U/ml	4 U/ml	8 U/ml	10 U/ml
Medium	24.2 ± 2.5	21.8 ± 2.4	19.5 ± 1.9	15.1 ± 1.0	12.9 ± 1.7	12.5 ± 1.8
UCHL-1	25.5 ± 2.3	22.5 ± 1.9	17.5 ± 2.1	14.0 ± 1.1	11.5 ± 2.0	9.5 ± 1.7
GAP8.3	25.1 ± 2.0	20.5 ± 1.8	17.6 ± 1.8	15.5 ± 1.2	13.5 ± 1.9	11.5 ± 1.4

[a] Cytokine withdrawal induces apoptosis, which is not further augmented by CD45 signals. Lymphoblasts were generated as described. Four × 10^5 cells/well were incubated for 3 days with the reagents as indicated. Apoptosis was quantified as described in *Materials and Methods*. Numbers show mean percentages of apoptotic cells ± SD and stem from three different experiments.

(43, 44). Thus, based on the results as demonstrated, it was tempting to hypothesize that CD45 phosphotyrosine phosphatase interferes with these phosphorylation processes, thereby limiting cellular activation through cytokine signals. To test this, we performed Western blots, staining with Abs recognizing the tyrosine-phosphorylated forms of STAT-3, STAT-5, STAT-6, or ERK1/2. As control for equal protein loading, we used Abs detecting all forms of the respective protein after stripping of the identical membranes. As shown in Fig. 3, compared with cells kept in medium (*lanes 1–3*), IL-2 induced tyrosine phosphorylation of STAT-3, STAT-5, and ERK1/2 (*lane 4*), whereas IL-4 led to phosphorylation of STAT-3 and STAT-6 (*lane 7*). Notably, however, addition of the CD45R0 mAb UCHL-1 or the pan-CD45 mAb GAP8.3 (Fig. 3, *lanes 2* and *3*) was ineffective alone. UCHL-1 strongly diminished IL-2-mediated phosphorylation of STAT-3, STAT-5, and ERK1/2 (Fig. 3, *lane 5*), and IL-4 induced phosphorylation of STAT-3 and STAT-6 after IL-4 stimulation (Fig. 3, *lane 8*). Control mAb GAP8.3 did not significantly influence STAT or ERK phosphorylation under these conditions (Fig. 3, *lanes 3, 6,* and *9*). Importantly, after stripping of the membranes as described in *Materials and Methods*, staining with STAT or ERK Abs recognizing all forms of these intracellular signaling molecules revealed equal protein loading in each lane (with some lower protein content in the STAT-3 blot in *lane 9*).

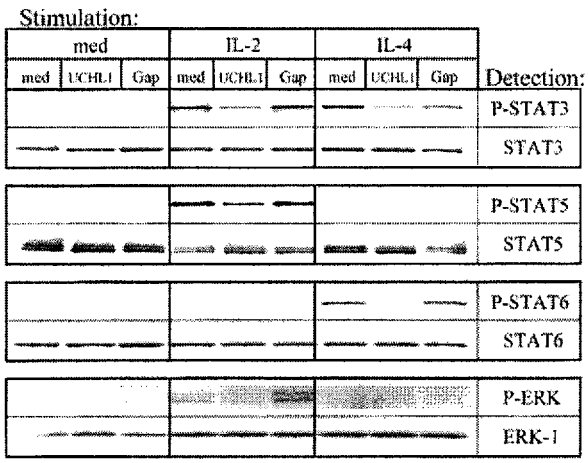

FIGURE 3. CD45R0 signals influence phosphorylation of STAT and ERK1/2 molecules. Lymphoblasts were generated as described. After 8 h of cytokine deprivation, UCHL-1 or GAP8.3 (Gap) mAb was added as specified. After 10 min, IL-2 or IL-4 was pipetted to culture medium, and cells were lysed after another 10–20 min. Western blots for phosphorylated STATs or ERK1/2 were performed as described in *Materials and Methods*. Thereafter, membranes were stripped, washed, and exposed to mAbs recognizing all forms of STATs or ERK1/2. The experiment shown is representative of three different experiments with similar results.

Next, we investigated whether CD45R0 signals not only influenced DNA synthesis and early intracellular cytokine signaling, but could also change mRNA production after IL-2 or IL-4 stimulation. Fig. 4 denotes that stimulation of lymphoblasts with IL-2 (*lane 4*) specifically led to increased IL-13 mRNA synthesis, with no effects on IL-2, TNF-α, IFN-γ, or IL-5 mRNA. IL-4 was not effective in inducing mRNA synthesis over the background level. Interestingly, under costimulation with IL-2 or IL-4, UCHL-1 (Fig. 4, *lanes 5* and *8*), but not GAP8.3 signaling (Fig. 4, *lanes 6* and *9*) alone could consistently diminish IL-2 mRNA quantities at least 4-fold (semiquantified as described by Lagoo-Deenadayalan et al. (40)). This was also seen in many, but not all lymphoblasts only stimulated with UCHL-1 (Fig. 4, *lane 2*). Moreover, coactivation through UCHL-1 and IL-2 and, to a lesser extent, GAP8.3 and IL-2, but not Ab combinations along with IL-4, increased TNF-α mRNA levels (Fig. 5, *lanes 3–6* and *7–9*). Furthermore, we investigated the influence of the various stimuli on mRNA expression of IL-4, TNF-α receptors p60 and p80, and IL-12. IL-4 and TNF-α receptor p80 mRNA levels were unaffected, whereas we could not detect IL-12 or TNF-α receptor p60 mRNA in lymphoblasts (data not shown).

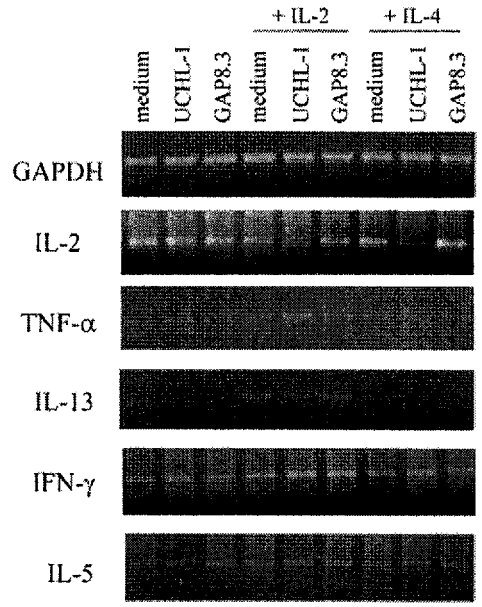

FIGURE 4. Influence of CD45 signals on mRNA quantities in lymphoblasts. Lymphoblasts were generated as described. After stimulation under the various conditions as specified, cells were lysed, RNA isolated, mRNA reverse transcribed, and amplified as described in *Materials and Methods*. The results shown are representative of three different experiments after PCR amplification for 28 cycles.

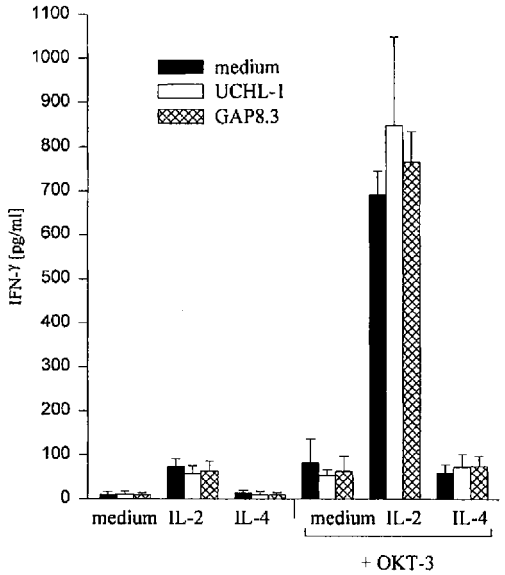

FIGURE 5. Influence of CD45 signals on IFN-γ secretion by lymphoblasts. Lymphoblasts were generated as described. Sixteen to 20 h after stimulation under the various conditions as specified, supernatants were collected and concentrations of IFN-γ were determined as described in *Materials and Methods*. Data are mean values ± SD from three different experiments.

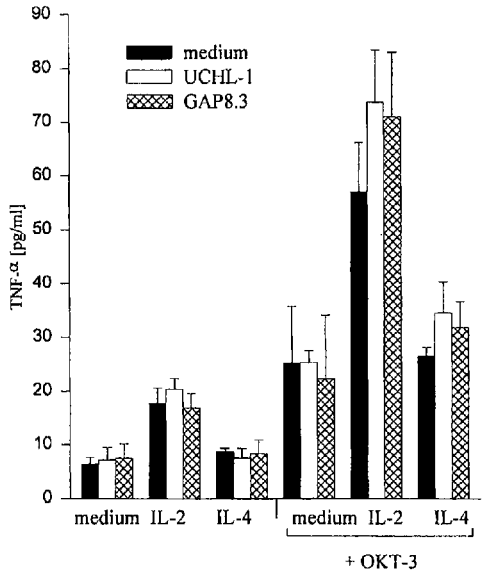

FIGURE 6. Influence of CD45 signals on TNF-α secretion by lymphoblasts. Lymphoblasts were generated as described. Sixteen to 20 h after stimulation under the various conditions as specified, supernatants were collected and concentrations of TNF-α were determined as described in *Materials and Methods*. Data are mean values ± SD from three different experiments.

Similarly, we wondered, whether these changes in mRNA production were also reflected in alterations of protein secretion. To this end, we collected supernatants after overnight stimulation of lymphoblasts under identical experimental conditions. We did not detect IL-2 or IL-4 in any supernatants (except for those where the cytokines had been added exogenously, data not shown). Measurements of IFN-γ, TNF-α, and IL-5 concentrations indicated that IL-2, but not IL-4, was able to stimulate secretion of the cytokines in low quantities over the background level, which was not altered by signals through CD45 epitopes recognized by UCHL-1 or GAP8.3 (Figs. 5–7). In contrast, after activation through CD3, lymphoblasts secreted TNF-α and IFN-γ, but not IL-5, in quantities comparable to the IL-2 effects (Figs. 5–7). Secretion of these cytokines was again not significantly influenced by CD45 cosignaling. Costimulation with OKT-3 and IL-2, but not OKT-3 and IL-4, consistently and synergistically induced secretion of high levels of TNF-α (Fig. 6) and especially IL-5 and IFN-γ (Figs. 5 and 7), which was again not diminished by CD45 signaling.

Discussion

In this paper, we investigated the role of CD45 signaling in activated human lymphoblasts. As described, lymphoblasts are not comparable to quiescent lymphocytes in their intracellular signaling responses to the identical stimuli. For example, Fas/Apo-1 stimulation will lead to apoptotic cell death in activated cells, whereas quiescent lymphocytes are unresponsive to these triggers (45). CD3 stimulation in quiescent cells leads to cellular proliferation; in activated cells, however, these triggers can lead to apoptotic cell death under certain circumstances (46). Since we have been studying intracellular CD45 signaling in quiescent PBMCs in the past (3, 37, 38), we were interested in the effects of these stimuli in activated lymphoblasts.

We herein describe that signals through CD45R0 can inhibit proliferation, mRNA synthesis, and γ_c receptor-associated signals in human peripheral nonlineage lymphoblasts. However and importantly, signals through the epitopes recognized by pan-CD45 mAb GAP8.3 have different effects in lymphocytes vs lymphoblasts: in quiescent cells this mAb is most effective in inhibiting OKT-3-mediated proliferation (Fig. 1), whereas it served as a negative control mAb in our experiments with lymphoblasts, not influencing γ_c- and OKT-3-induced signals. These data argue for a cell cycle-dependent function of certain CD45 epitopes in human

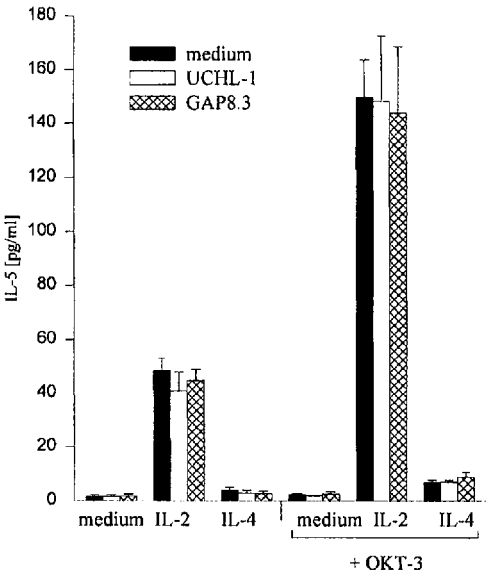

FIGURE 7. Influence of CD45 signals on IL-5 secretion by lymphoblasts. Lymphoblasts were generated as described. Sixteen to 20 h after stimulation under the various conditions as specified, supernatants were collected and concentrations of IL-5 were determined as described in *Materials and Methods*. Data are mean values ± SD from three different experiments.

lymphocytes. One could speculate that CD45 association to signaling rafts differs in lymphocytes vs lymphoblasts, and that the various CD45 epitopes differentially regulate intracellular signaling complexes present in the adjacent signaling rafts. These findings again highlight the unique role of CD45 among cellular surface molecules.

CD45R0 mAb UCHL-1 showed similar efficacy in limiting γ_c cytokine-mediated lymphoblast proliferation independent of the cytokine used. Vice versa, cosignaling through OKT-3 was more effective in preventing UCHL-1-mediated inhibition of IL-4- or IL-7-stimulated cell growth. In experiments studying intracellular signaling events, we found that signaling through CD45R0 specifically inhibited IL-2-triggered phosphorylation of STAT-3, STAT-5, and ERK-1/2 as well as IL-4-stimulated phosphorylation of STAT-3 and STAT-6. STAT proteins are activated through members of the Janus kinase (Jak) family (Jak1, Jak2, Jak3, Tyk2) which play a crucial role in many cellular functions (47, 48). Based on our findings, one could speculate that CD45 signals regulate STAT phosphorylation through differential activation of Jak1 or Jak3 in human lymphoblasts. Indeed, Irie-Sasaki et al. (49) have recently shown that in CD45-negative cell lines (lymphocytic, monocytic, mast cells) and mice the cell-associated members of the Jak family as well as STAT-1, STAT-3, or STAT-5 are hyperphosphorylated. Moreover, the authors provide evidence for a direct association of Jak2 with the intracellular phosphatase domains of CD45 in vitro, leading to dephosphorylation (49). These findings clearly support our hypothesis of a regulation of STAT phosphorylation by CD45 signals (as shown in this paper) through a direct deactivation of Jak1 and/or Jak3 in human lymphoblasts.

PHA-stimulated and IL-2-expanded lymphoblasts readily undergo apoptosis when cultured in the absence of γ_c cytokines (41). Moreover, signaling through CD95, steroid receptors, or addition of chemotherapeutic agents like daunorubicine or etoposide leads to programmed cell death in a large proportion of these cells (50). Thus, we hypothesized that inhibition of proliferation in these highly activated cells must consequently lead to apoptosis. However, as shown in *Results*, despite considerable inhibition of proliferation we did not see induction of programmed cell death over the background level in these apoptosis-prone lymphoblasts in response to CD45 signals. We could recently confirm these results in experiments in which we almost completely suppressed cytokine-mediated proliferation in lymphoblasts by addition of agents inhibiting various intracellular signaling kinases. Under those conditions we did not find a relevant increase of apoptotic cell death (Ref. 51; C. Gabler, T. Hieronymus, N. Blank, M. Schiller, J. H. Berden, S. Winkler, J. R. Kalden, and H.-M. Lorenz, manuscript in preparation). These data support the notion that regulation of proliferation and apoptosis are not necessarily interrelated.

As shown in this paper, human lymphoblasts display a dual role which is differentially regulated. On the one hand, there is proliferation and survival which is dependent on exocrine stimulation with γ_c cytokines (we found no autocrine production of IL-2 and IL-4 by lymphoblasts). This response is mediated through Jak/STAT-directed signaling events and is controlled by CD45 stimuli as demonstrated in this paper. On the other hand, there is production of cytokines which clearly do not contribute to proliferation and survival (Ref. 41 and C. Gabler, T. Hieronymus, N. Blank, M. Schiller, J. H. Berden, S. Winkler, J. R. Kalden, and H.-M. Lorenz, unpublished observation) and might stimulate neighboring APCs or lymphocytes/lymphoblasts (TNF-α, IFN-γ, IL-5). However, synthesis of these cytokines is supported by signals through the γ_c cytokine receptors and TCR-CD3 complex, but, in contrast to the proliferative response, cannot be inhibited by CD45 signals (Figs. 6–8), which suggests that secretion of these cytokines occurs independent of STAT/ERK signaling molecules.

Our data collectively associate γ_c cytokine signaling events with CD45-regulated intracellular signaling processes in nonlineage human lymphoblasts and again highlight the dominant role for CD45 in influencing early cellular activation steps in human lymphocytes and lymphoblasts. Recently, CD45 was discussed as membranous gatekeeper determining early intracellular signaling events by influencing phosphorylation or dephosphorylation of associated proteins organized in lipid microdomains (35). Extraction of plasma membrane proteins with certain nonionic detergents at cold temperatures results in segregation of constituents into soluble and insoluble fractions. The insoluble fraction is enriched in cholesterol, glycosphingolipids, membrane proteins linked to glycophosphatidylinositol, and certain signaling proteins including src family kinases, CD3/TCR, CD4 or CD8, and linker for activation of T cells (35, 52–54). It was concluded that signaling molecules differentially and specifically translocate into membrane microdomains after activation. The role of CD45 in controlling signaling processes through these microdomains is still disputed. It has previously been noted that the extracellular domain of CD45 is much larger in size when compared with the Ag receptor and low molecular mass signaling molecules (35). On the other hand, CD45 has been shown to be recruited to the contact area of a T cell with an APC (55, 56). Thus, it was hypothesized (35, 36) that CD45 is excluded from membrane microdomains, but must be in close proximity to the signaling rafts. In this context, it will be of great interest to investigate formation of microdomains in lymphokine-stimulated lymphoblasts and to study the differential role of CD45-phosphatase in influencing signaling processes in these lymphoblasts.

Acknowledgments

We are grateful to Drs. P. Beverly, J. Byrne, B. Schraven, and S. Meuer for generously providing Abs and hybridoma cells as stated in *Materials and Methods*.

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Am J Dermatopathol. 2010 Apr;32(2):183-6.

When dead cells tell tales-cutaneous involvement by precursor T-cell acute lymphoblastic lymphoma with an uncommon phenotype.

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Abstract

The thymic type of precursor T-cell acute lymphoblastic lymphoma (pre-T ALL), an uncommon T-cell malignancy, typically presents as a thymic mass and expresses terminal deoxonucleotidyl transferase, CD7, and cytoplasmic CD3, with variable expression of other markers. Cutaneous presentation in pre-T ALL is highly unusual. We describe a case of pre-T ALL presenting as 2 papulonodular lesions on the face of an otherwise asymptomatic 27-year-old man. Microscopic examination of both lesions revealed a moderate to dense pandermal infiltrate of medium-sized lymphocytes with extensive "crush" artifact, whereas immunohistochemistry revealed positive staining of lesional cells for CD45, CD3, Bcl-2, Ki-67, CD5, CD7, and CD34 but negative staining for CD4, CD8, CD30, CD56, CD10, CD117, anaplastic lymphoma kinase protein, TdT, myeloperoxidase, CD79a, and CD20. Gene rearrangement studies performed on both biopsies identified a clonal population of T lymphocytes. A subsequent computed tomography scan revealed a 9-cm mediastinal mass encasing all major mediastinal vessels, whereas a bone marrow biopsy revealed blasts with an immunophenotype similar to that of the cutaneous lesions. Features unique to this case include the cutaneous presentation and the immunophenotype-absence of CD4, CD8, and TdT with expression of CD34-both highly unusual features for pre-T ALL.

PMID: 20010405 [PubMed - indexed for MEDLINE]

Leuk Res. 2008 Feb;32(2):263-7. Epub 2007 Aug 9.

CD45 expression in low-grade B-cell non-Hodgkin's lymphomas.

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Abstract

CD45 is a glycoprotein expressed in all lymphohemopoietic cells. Its expression increases during B-lymphocyte ontogeny. Few data are available about CD45 expression in the various types of low-grade B-cell non-Hodgkin's lymphomas (NHL). Low levels of CD45 have been reported in pathologic lymphocytes from typical chronic lymphocytic leukemia (CLL) and higher levels of this antigen have been observed in some cases of atypical CLL and in some cases of other types of NHL. One hundred and seven bone marrow samples of NHL with bone marrow infiltration were investigated: 45 typical CLL, 15 atypical CLL, 9 mantle cell lymphomas (MCL), 1 MCL with CD23 expression, 18 marginal zone lymphomas (MZL), 6 lymphoplasmacytic lymphomas (LPL), 6 follicular lymphomas (FL), and 7 hairy cell leukemias (HCL). CD45 expression was evaluated by flow cytometry: pathologic lymphocytes were identified on the basis of specific immunophenotypic profile, CD19/K or CD19/lambda co-expression. Results were expressed as median fluorescence intensity (MFI) along a 1024 linear scale. CD45 expression was measured also on autologous T-lymphocytes and a "CD45 index" was calculated as the ratio MFI of pathologic B-lymphocytes/MFI of T-lymphocytes, to normalize the results obtained. We found four CD45 expression patterns: very low in typical CLL; relatively low in MCL; intermediate intensity in MZL, LPL, and FL; very high expression in HCL. Among the atypical cases, very high CD45 expression was found in one case of CD23-negative CLL, in CD23-positive MCL, and CLL with atypical morphology. The results indicate different levels of maturation in low-grade NHL and may help to characterize such neoplasias.

PMID: 17692374 [PubMed - indexed for MEDLINE]